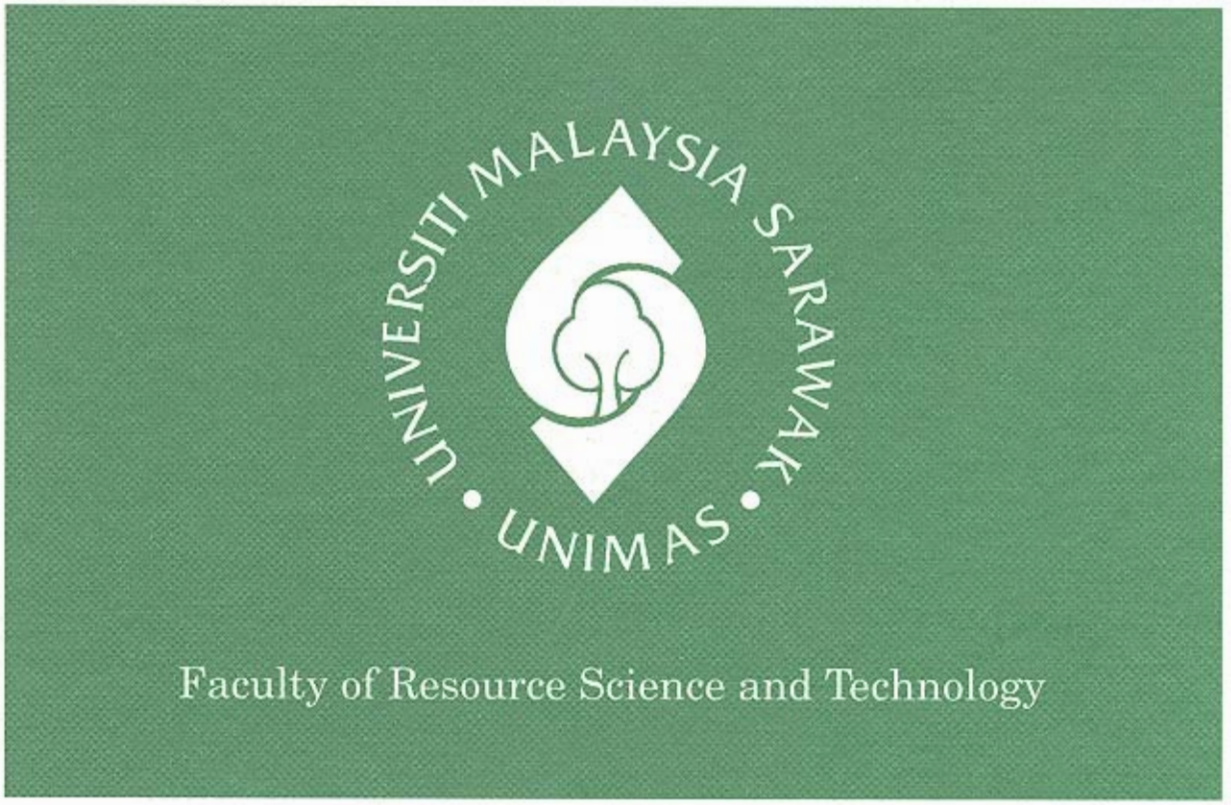


# EFFECTS OF NATURAL RUBBER SERUM POWDER (NRSP) AND NATURAL RUBBER SERUM CONCENTRATES (NRSC) AS ALTERNATIVES TO YEAST EXTRACT FOR LACTIC ACID FERMENTATION

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# EFFECTS OF NATURAL RUBBER SERUM POWDER (NRSP) AND NATURAL RUBBER SERUM CONCENTRATES (NRSC) AS ALTERNATIVES TO YEAST EXTRACT FOR LACTIC ACID FERMENTATION

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the requirements for the degree of Bachelor of Science with Honours
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# Effects of Natural Rubber Serum Powder (NRSP) And Natural Rubber Serum Concentrates (NRSC) As Alternatives to Yeast Extract For Lactic Acid Fermentation

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## ABSTRACT

Lactic acid production from fermentation by *Lactococcus lactis* IO-1 of hydrolysed sago starch was done by using five different fermentation media components (bacterial growth promoters): 5 g/L yeast extract, 5 g/L NRSP, 5 g/L NRSC, 5 g/L NRSP + 5 g/L yeast extract and 5 g/L NRSC + 5 g/L yeast extract and medium 0 g/L promoters act as control. The research objective was to compare which component can promote optimal production of lactic acid, and this is defined by glucose consumed by the bacteria and biomass produced. From observation, batch fermentation trials utilizing 5 g/L yeast extract had produced higher biomass than 0 g/L promoters by the difference of 0.5 g at 12 hours. Meanwhile, glucose consumption and dry cell weight of fermentation products with other promoters' exhibit inaccurate results due to the promoters (NRSP and NRSC) were not dissolved in the medium. However, data from glucose consumption efficiency and Y x/s shows that NRSC have the potential for lactic acid fermentation, if only further treatments are done to make it media soluble. All fermentations were run for 24 hours at 37°C and shaking at 150 rpm.

Key words: Batch fermentation, *Lactococcus lactis* IO-1, yeast extract, NRSP, NRSC.

## *ABSTRAK*

*Penghasilan asid laktik daripada fermentasi kanji sagu terhidrolisis oleh Lactococcus lactis IO-1 telah dikaji menggunakan lima komponen media fermentasi (penggalak pertumbuhan bakteria) yang berbeza: 5 g/L ekstrak yis, 5 g/L NRSP, 5 g/L NRSC, 5 g/L NRSP + 5 g/L ekstrak yis and 5 g/L NRSC + 5 g/L ekstrak yis dan media dengan 0g/L penggalak bertindak sebagai kawalan. Kajian ini bertujuan membuat perbandingan komponen yang akan menghasilkan asid laktik secara optimum, dan ini dibuktikan dengan glukosa yang digunakan oleh bakteria dan biomas yang dihasilkan. Daripada pemerhatian, fermentasi kelompok yang menggunakan 5 g/L ekstrak yis menghasilkan biomas yang lebih tinggi daripada media dengan 0 g/L penggalak dengan perbezaan 0.5 g pada jam ke-12. Manakala penggunaan glukosa dan berat sel kering daripada produk fermentasi yang menggunakan penggalak yang lain menunjukkan keputusan yang tidak tepat kerana penggalak-penggalak tersebut (NRSC dan NRSP) tidak larut dalam medium fermentasi. Namun begitu, data keefisienan penggunaan glukosa dan Y x/s menunjukkan NRSC mempunyai potensi untuk fermentasi asid laktik, cuma memerlukan rawatan lanjutan untuk menjadikannnya larut dalam media. Semua fermentasi dijalankan selama 24 jam pada suhu 37°C dengan 150 rpm.*

*Kata kunci: Fermentasi kelompok, Lactococcus lactis IO-1, ekstrak yis, NRSP, NRSC.*

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Natural rubber latex (NRL) is a whitish, milky liquid derived by tapping the bark of *Hevea brasiliensis*, commonly known as the rubber tree. NRL comprise 30-40% of the rubber hydrocarbon particles suspended in the serum together with a few percent of other non-rubber substances such a proteins, lipids, carbohydrates, sugar, some metals (non-rubber fraction) and the remaining major component is water. The approximate composition of latex is shown in the table below (Patel *et al*, 2000)

Table 1: Latex components and the approximate proportions.

| **Component** | % |
|---|---|
| Rubber | 30.0-40.0 |
| Protein | 2.0-2.5 |
| Organic matter | 2.0-3.0 |
| Ash | 0.7-0.9 |
| Moisture | 55.0-60.0 |

Natural Rubber Serum (NRS) is actually a problematic liquid waste generated during the processing of latex into rubber sheets (Muari, 2000) due to the high contents of

nitrogenous compound. However, as tested in laboratory, Natural Rubber Serum Concentrates (NRSC- a concentrates derived from processed natural rubber serum) was tested as a growth stimulator on *Saccharomyces cerevisiae*. Besides, when NRSC converted into Natural Rubber Serum Powder (NRSP), this compound has been shown to possess favourable growth factors (Oiki *et al*, 1996; Bujang *et al*, 1997, Bujang *et al*, 1998) containing high amount of protein and free amino acids (Ishizaki, 1995). Previous research have shown the possibilities of using natural rubber serum powder (NRSP) or natural rubber serum concentrate (NRSC) as an alternative to yeast extract (Bujang *et al*, 2005). In addition, from a study done by Muari, 2000, it was concluded that NRSC is a better growth stimulator for *S. cerevisiae* compares to yeast extract.

Therefore, in this project, the same idea will be applied with some modifications as *Lactococcus lactis* IO-1 will be used instead of *S. cerevisiae* and NRSP and NRSC will be used, obtained by converting NRS into powder form using a spray-dryer to produce NRSP and using rotary evaporator to evaporate ethanol from NRS mixture to produce NRSC.

## 1.2 Objectives

This study was carried out in order to convert NRS into NRSP and NRSC, to study the growth of *L. lactis* IO-1 and to gain knowledge on the potential of NRSP and NRSC to replace yeast extract for production of lactic acid. The main concerns of this project are stated below:

i. To compare the effects of NRSP and NRSC instead of yeast extract on the bacterial growth based on Dry Cell Weight (DCW) /biomass analysis of *L. lactis* IO-1.

ii. To compare the utilization of glucose by *L. lactis* IO-1 when NRSP and NRSC is used instead of yeast extract in fermentation.

iii. To exhibit the efficiency of NRSP and NRSC compared to yeast extract for lactic acid production.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Natural Rubber Serum (NRS)

Natural rubber serum is a waste product of the rubber industries generated during the processing of latex into rubber sheets. It contains high level of sugar and protein that might react with bacteria or other microorganisms in biodegradation of organic matter producing offensive smell. Apart from that, the high contents of ammonium and sulphate might negatively affect aquatic flora and fauna, and therefore the improper management of natural rubber serum will bring to the water pollution. To make it worst, discarding this non-rubber waste product either biologically or via biochemical method is expensive and not suitable in term of ecology (Patel *et al*, 2000)

## 2.2 Natural Rubber Serum Concentrates (NRSC)

According to Muari (2001), natural rubber serum can be purified to produce Natural Rubber Serum Concentrates (NRSC), that have the ability to stimulate the growth of certain type of microorganism and in the fermentation industries for ethanol production (Tripechkul *et al*, 1992).

NRSC produced in the laboratory was tested as growth stimulator on *S. cerevisiae*. It was revealed that the addition of 6% of NRSC (2.2g NRSC) in 100 ml nutrient broth could stimulate or encourage the growth of this yeast better, with an increased dry weight of 79.68% (Initial dry weight was 15.01 g/L and increased to 26.97 g/L after 30 hours). Meanwhile, the growth of yeast in the medium without NRSC could only reach an increased dry weight of 33.33 % only. It was concluded that NRSC is a better growth stimulator for *S. cerevisiae* compares to yeast extract (Muari, 2001). In this project, instead of using *S. cerevisiae*, *Lactococcus lactis* IO-1 is being used.

## 2.3 Natural Rubber Serum Powder (NRSP)

Beside purification of NRS into NRSC, NRS also can be converted into powder form that is termed as Natural Rubber Serum Powder in a process that use spray-dryer to evaporate the containing ethanol in the NRS. As NRSC had produced such encouraging growth in *S. cerevisiae*, it is expected that NRSP will have a similar performance although *L. lactis* IO-1 is being used instead of *S. cerevisiae*.

## 2.4 Sago Starch

The state of Sarawak produce large amount of sago starch generating an usual income of over RM42.2 million (DoS, 1999). Production of lactic acid has been designated as the most economical venture for sago starch fermentation in enhancing the commercial value of sago starch with the least environmental effects (Ishizaki, 1997). Sago starch is a cheap

and locally available compound as carbon source for production of lactic acid (Bujang, 2005). Such characteristics of carbon source is needed to minimized the production cost lactic acid therefore sago starch is also being chose in this project to serve the same purpose.

## 2.5 *Lactococcus lactis* IO-1

*Lactococcus lactis* IO-1 is an AT-rich, nonsporulating, facultatively anaerobic nonmotile bacterium that group in pairs and short chain. Their typical size is 0.5-1.5 μm in length. This species of bacteria commonly found on plants, animal skin and hair and being used extensively in the production of buttermilk and cheese because the curdle and perfumed milk (Anon., 2004)

According to Ishizaki *et al* (1990), pH 6.0 and 37°C is the optimum condition for *L. lactis* IO-1 to grow. Mentioned strain is gram- positive, ovoid coccus and catalase negative. The strain fermented various carbohydrates to produce L-lactate with high conversion rate and no other volatile fatty acid was detected. This is important as only L- lactate is needed as the product of the fermentation that is going to be done.

## 2.6 Lactic Acid

Lactic acid is the mostly occurring carboxylic acid in nature. The Swedish chemist, Scheele, first discovered it in 1780, but it was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Narayanan, 2004). Scientifically, lactic acid is a carboxylic acid and an alcohol; its chemical formula is $C_3H_6O_3$ and its structure is reflected in its systematic name 2-Hydroxipropanoic acid. It exhibits low volatility and its solution; it can loose a proton from the COOH carboxy group, turning into the lactate ion $CH_3CHOHCOO^-$ (Narayanan, 2004).

Besides being produced via natural synthesis in our muscles, this lactic acid also can be produce fermentatively. Fermentative production has some advantages. For instance, by choosing a lactic acid bacterium producing only one of the isomers, an optically pure product is obtained. It is also possible to use renewable resources as substrates, such as starch and cellulose (Hofvendahl, 1969). As in my case, sago starch will be used for fermentation medium.

Lactic acid has been produced commercially by fermentation since 1883. Among the major application of lactic acid is in the food industry as an additive and preservative. Other application includes usage as a pharmaceutical intermediate, lactate ester, which is an alternative solvent to glycol ether (Li, 2003). However, all this potential can only be realized if the cost of production is competitive on a global scale. Therefore, for my research, NRSP and NRSC as a new source that is derived from rubber waste product.

## 2.7 Spray-Dryer

Spray drying allows different liquid components to be blended and dried in one continuous step. A renewed interest in powder properties such as particle size and bulk density has made equipment selection an important consideration in today's ceramic labs and pilot plants (Andrew, 2005).

A typical laboratory spray drying system starts with a heated air source that is introduced into a drying chamber along with a liquid feed. An atomizer is used to break the liquid feed into a spray or mist so that the hot air can evaporate the liquid, leaving the feed in a dry particulate form. The dried particles and moisture-laden air pass from the drying chamber into the powder separator, which is usually a cyclone, bag house or a combination of the two. The dried particles are discharged from the bottom of the cyclone, while the air is pulled out of the top and is released to the atmosphere or sent to a bag house separator for further cleaning (Andrew, 2005).

## 2.8 Rotary Evaporator

The rotary evaporator is used for the rapid removal of solvents by distillation at reduced pressure. This equipment is distinctive by the fact that the distillation flask is rotated in an automatically-controlled-temperature waterbath during the removal of solvent. This rotating movement performs the two important functions of reducing the risk of bumping, and increasing the rate of volatilization of solvent by spreading the contents around the

walls of the flask in a thin film. To use the rotary evaporator, the evaporating flask is filled with solution. The waterbath will heat up the solution to the desired temperature. The evaporating solution will pass through a chamber into a condenser, with cold water passing through the condenser coil and the condensed solvent is collected in a receiving flask (ACS, 2002).

## 2.9 Batch Fermentation

Batch fermentation can be considered as a system that represents growth in a closed system using shake flask or bioreactor. In batch processes, the reactor is filled with a sterile nutrient substrate and inoculated with the microorganism. The culture is allowed to grow until no more of the product is being made. Besides that, all nutrients required during one run of cultivation except for molecular oxygen in an anaerobic process and ammonia or other chemicals for pH adjustment, are added to the medium before cultivation started, and the broth containing the product is withdrawn only at the end of each batch run (Yamane, 1995). According to Jolherry (2002), biomass or dry cell weight is an important parameter in batch fermentation processes.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 MATERIALS:

### 3.1.1 Sago Starch

Food grade sago starch powder was obtained from the local market.

### 3.1.2 Latex

Latex used in this research was provided by Institut Pertanian Sarawak (IPS), Semenggok, Kuching.

### 3.1.3 Microorganisms

The microorganism to be used in the experiment is *Lactococcus lactis* IO-1, a Japanese Collection of Microorganism (JCM) 7638.

### 3.1.4 Enzymes for Hydrolysis

The enzymes used for hydrolysis were termamyl-120L (thermostable amylase from *Bacillus lincheniformis*, 120 KNU/g) and Dextrozyme (a mixture of glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*, 225 AGU/ml) supplied by Novo Nordisk.

### 3.1.5 Fermentation Medium

#### 3.1.5.1 Fermentation Medium with Yeast Extract

The basal medium for fermentation was glucose broth consisting of 5 g/L polypeptone (Difco, USA), 5 g/L yeast extract (Difco, USA), 5 g/L natrium chloride (UNIVAR, Australia) and distilled water. Glucose concentration for the initial batch phase was 60 g/L. Similar medium containing 10 g/L glucose was used for inoculum preparation. The 10 % (v/v) inoculum was used for all fermentation trial (Ishizaki and Ohta, 1989).

#### 3.1.5.2 Fermentation Medium With NRSP and NRSC

To study the effect of NRSP and NRSC on *Lactococcus lactis* IO-1, yeast extract was replaced by NRSP and NRSC. A few similar fermentation mediums (such in 3.1.5.1) was prepared but yeast extract was replaced with 5 g/L NRSP, 5 g/L NRSC, 5 g/L yeast extract + 5 g/L NRSP and 5 g/L yeast extract + 5 g/L NRSC.

### 3.1.6 Culture Systems

Fermentation was carried out in a 250 ml shake flask with 200 ml working volume. The agitation speed being used was 150 rpm and temperature maintained at 37°C. The culture pH was adjusted at pH 6.0 by addition of 10 M NaOH at the initial stage of the experiment.

## 3.2 METHODS

### 3.2.1 Production of Natural Rubber Serum (NRS)

Natural rubber latex was added to ethanol in a 1:1 ratio. This mixture then transferred into a disclosed vessel and subjected to shaking to 150 revs/ min at 37°C using a shaker for overnight. The pieces of latex due to the coagulation process were taken out from the vessel and being discarded. Secondly, the solution was kept in refrigerator for overnight as a further step to separate the remaining rubber particle from serum (rubber particle coagulate when kept in a low temperature). Next, this solution was subjected to filtration again to filter the remaining rubber particle. The filtered solution that is also known as NRS was then used to produce Natural Rubber Serum Concentrates and Natural Rubber Serum Powder.

### 3.2.2 Production of Natural Rubber Serum Powder (NRSP)

NRSP was produced by running the NRS in a spray-dryer to evaporate the ethanol and water in order to gain the serum in the form of powder. The NRSP and NRSC obtained, later, will be used in fermentation process to study its effect on *Lactococcus lactis* IO-1. The idea is to grow *L. lactis* IO-1 in a medium containing yeast extract and other medium containing different concentration of NRSP and NRSC with the aim to compare between yeast extract and NRSP and NRSC effect on the growth of *L. lactis* IO-1.

### 3.2.3 Production of Natural Rubber Serum Concentrates (NRSC)

NRSC was produced by evaporating the ethanol in the NRS at 80°C by using the rotary evaporator. The concentration of the NRSC produced is 50 times more concentrated than the NRS (this means that 1L NRS was used to produced 20 ml NRSC). The NRSC produced was filtered with filter paper no.2 to remove remaining coagulation of latex, before being used in fermentation.

### 3.2.4 *Lactococcus lactis* IO-1 Activation

*Lactococcus lactis* IO-1 was taken out from stock culture (-80°C) and was left at room temperature. The microorganism then being transferred to Thioglycolate (TGC) medium without dextrose and was incubated for 18 hours at 37°C and pH 6.0 for maximum

growth rate (Ishizaki *et al*, 1990). The 2 ml starter culture was then transferred to 18 ml seed culture at pH 6.0 in a universal bottle and incubated at 37°C for another 6 hours.

### 3.2.5 Enzymatic Hydrolysis of Sago Starch

100 µl of Termamyl 120-L and 120 µl of Dextrozyme is used for this process. 200g of sago starch was added into 1L of distilled water and then pH being adjusted to pH 6.5. The mixture then being heated up to 90-100°C for 10 minutes and this stage is called gelatinization. 100µl of Termamyl being added to the mixture together with 6mg of Calcium ion during liquefaction stage at ph 6.5. Then the temperature was maintained at 80- 90°C for 2 hours. Next, 120µl of Dextrozyme was added during the saccharification stage. The mixture's temperature was maintained at 60-65°C with pH 4.5 for 4 hours. After completed, the hydrolyzed sago starch was being centrifuged prior to fermentation process.

### 3.2.6 Sampling

Each flask of fermentation was operated until 24 hours. Sampling was done every 6 hours starting from 0 hours, 6 hours, 12 hours, 18 hours and 24 hours. For each sampling, 10 ml were taken manually from the culture system and placed in universal bottle. The samples were kept at low temperature (4°C) before being analyzed.

### 3.2.7 Analytical techniques

#### 3.2.7.1 Glucose Determination

Glucose (from hydrolyzed sago starch), which was used as carbon source in fermentation, was utilized by the microorganisms throughout the fermentation. Reduction of glucose was determined using Dinitrosalycylic (DNS) method (Miller, 1959). Refer appendix 2 for further details of DNS mixture preparation. For glucose determination, 3 ml of DNS mixture and 3 ml of sample were put in to a test tube, then boiled (in a boiling water) for 15 minutes. After left for cooling, 40% of Rochelle salt was added and the OD value was determined by UV spectrophotometer with the $\lambda$ at 575 nm.

#### 3.2.7.2 Dry Cell Weight Determination

Biomass concentration is measured based on dried cell weight (DCW) method. 10 ml of sample was centrifuged at 8000 rpm for 15 minutes at 4°C in a centrifuge tube (weight known). The supernatant then collected while the cells were suspended in sterile water. The cells were centrifuged again at 8000 rpm for 15 minutes at 4°C and then the cell free water was discarded. The cells will be dried in oven at 80°C overnight, until the weight is constant. DCW will be determined as follows:

$$\text{Dry Cell Weight (g/l)} = \frac{\text{Weight of dried tube + cells (g)} - \text{Weight of tube (g)} \times 10^3}{\text{Sample volume (ml)}}$$

# CHAPTER 4

# RESULTS AND DISCUSSIONS

## 4.1 Effects of Different Media Components (Bacterial Growth Promoters) On Biomass and Glucose Consumption (first trials).

Initially, fermentation trials were done with NRSP and NRSC (five times concentrations) with 100 ml working volume for 24 hours. Unfortunately, these had come unsuccessful as there were no pellet formed and the biomass produced was still suspended in the sample. To make things worse, the NRSP being used was not soluble in the media and the NRSC still contained latex coagulate. These had caused interference during sample analysis that lead to inaccurate results, both for dry cell weight determination and glucose consumption. Such problems were suspected to be caused by three important reasons.

Firstly, it was the working volume (100 ml) causing the biomass of *L. lactis* IO-1 not forming pellet at the bottom of centrifuge tube. This means not enough inoculums being supplied during the beginning of the fermentation, therefore not enough biomass being produced throughout the fermentation trials to form pellet when centrifugation was done. No pellet means dry cell weight determination could not be carried out. Upon no pellet was formed, the remaining cells that suspended in the sample had caused interference in absorbance reading and spectrophotometer test was unsuccessful.